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Chromosomal Inheritance of Epigenetic States in Fission Yeast During Mitosis and Meiosis

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Summary

Inheritance of the active and inactive states of gene expression by individual cells is crucial for development. In fission yeast, mating-type region consists of three loci called *mat1*, *mat2*, and *mat3*. Transcriptionally silent *mat2* and *mat3* loci are separated by a 15 kb interval, designated the *K*-region, and serve as donors of information for transcriptionally active *mat1* interconversion. In a strain carrying replacement of 7.5 kb of the *K*-region with the *ura4* gene, we discovered that *ura4* silencing and efficiency of mating-type switching were covariegated and were regulated by an epigenetic mechanism. Genetic analyses demonstrated that epigenetic states were remarkably stable not only in mitosis but also in meiosis and were linked to the mating-type region. This study indicates that different epigenetic states are heritable forms of chromatin organization at the *mat* region.

Introduction

Propagation of different states of gene expression over the course of multiple cell divisions is thought to be essential for promoting cellular differentiation (reviewed by Felsenfeld, 1992). The ways in which these states are established and propagated at each cell division remain fundamental questions in studies of development. Investigations of regulation of gene expression by position-effect control, a widespread phenomenon in eukaryotes, have provided important insights into this area of research. Position effect was first identified in Drosophila, whereby genes placed in close proximity to heterochromatin were unstably repressed (reviewed by Eissenberg et al., 1995). Such states of gene expression have been described as due to epigenetic events involving no alteration in DNA content. Several *trans*-acting modifiers of position-effect variegation have been identified and are thought to regulate expression indirectly by affecting heterochromatin formation (see Eissenberg et al., 1995). In budding yeast Saccharomyces cerevisiae and the fission yeast Schizosaccharomyces pombe, transcriptional repression at silent donor loci (reviewed by Laurenson and Rine, 1992; Klar, 1992), near telomeres (Gottschling et al., 1990; Nimmo et al., 1994) and centromeres (Allshire et al., 1994, 1995) closely parallel position-effect control observed in higher eukaryotes.

In fission yeast, the mating-type region consists of three loci called *mat1*, *mat2*, and *mat3*. *mat2* is located approximately 15 kb centromere distal to *mat1* and contains silenced *P* information, while *mat3* is approximately 15 kb distal to *mat2* and contains silenced *M* information (Beach, 1983; Beach and Klar, 1984). The interval between *mat2* and *mat3* is designated the *K*-region (Beach and Klar, 1984). Interestingly, no meiotic recombination has been observed in the *K*-region (Egel, 1984). Therefore, the *K*-region has been designated as a recombinational "cold spot." In contrast, in homothallic strains, designated h^{90}, the transfer of genetic information by gene conversion from silent *mat2* and *mat3* donor loci to transcriptionally active *mat1* occurs efficiently, a process that is initiated by the double-strand break at *mat1*, resulting in a switch of cell type (reviewed by Klar, 1992). The h^{90} cells switch to the opposite mating type in 72%–90% of the cell divisions (Miyata and Miyata, 1981), suggesting nonrandom utilization of donors. Thon and Klar (1993) have demonstrated that the location of the donor cassettes on the chromosome, rather than their genetic content, is the deciding factor for recognition of the donor for each cell type.

Mutations in the unlinked loci *clr1*, *clr2*, *clr3*, *clr4*, *swi6*, and *rik1* partially derepress the silent mating-type loci and also allow recombination in the *mat2–mat3* interval (Egel et al., 1989; Klar and Bonaduce, 1991; Lorentz et al., 1992; Thon and Klar, 1992; Ekwall and Ruusala, 1994; Thon et al., 1994). This indicates that transcription and recombination are blocked by the same mechanism. These six loci have also been shown to play a role in the silencing of the marker gene integrated adjacent to the telomeres and centromere 1 (*cen1*; Allshire et al., 1995). Most studies have proposed that *clr1*, *clr2*, *clr3*, *clr4*, *swi6*, and *rik1* gene products are involved, directly or indirectly, in the organization of heterochromatin-related structure at the *mat2–mat3* interval, centromeres, and telomeres, making these regions inaccessible to transcription factors and resulting in silencing and inhibition of recombination.

Here we demonstrate that an epigenetic mechanism regulates the efficiency of mating-type switching and silencing of marker genes inserted at the *K*-region. More importantly, these epigenetic states are chromosomally inherited during mitosis as well as during meiosis.

Results

ura4 Expression and Mating-Type Interconversion Are Covariegated in *KΔ ::ura4* Cells

Repression of the *ura4* marker gene placed adjacent to *mat2* and *mat3* suggests that silencing extends beyond the cassettes (Thon and Klar, 1992; Thon et al., 1994). To gain an insight into the role of *K*-sequences in silencing in this region, we replaced 7.5 kb of the *K*-region (see Figure 1) with the *ura4* marker gene to generate an h^{90}, *KΔ ::ura4* strain (SPG27; see Table 1 for complete genotype; Figure 1). Two results showed that the donor loci remained silent in the *KΔ ::ura4* cells. First, microscopic examination showed that haploid SPG27 cells grown on sporulation medium did not undergo haploid meiosis, which is defined as an aberrant event producing immature azygotic asci in haploid cells (Kelly et al.,

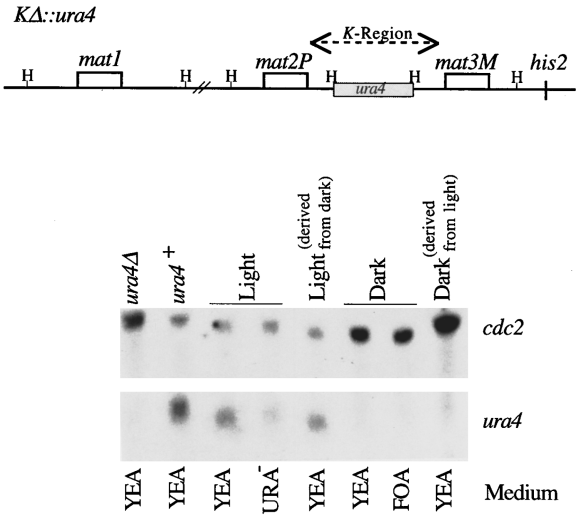

Figure 1. *KΔ ::ura4* Expression Is Correlated with Light and Dark Phenotypes

Line drawing at top shows the locations of the *mat1*, *mat2*, *mat3*, and tightly linked *his2* locus (not drawn to scale). H indicates the HindIII site. 7.5 kb of the *K*-region was replaced with *ura4* to construct *KΔ ::ura4* allele. The light and dark derivatives of h^{90}, *KΔ ::ura4* strain (SPG74) were isolated after growth on URA$^-$ and FOA media, respectively. However, the sporulation phenotype of the single isolated colonies grown on nonselective sporulation medium was used to identify secondary light and dark derivatives of original dark and light forms, respectively. Total RNA prepared from cultures grown in YEA, URA$^-$, or FOA medium at 33°C was subjected to Northern analysis, using *ura4* as the probe. Analysis of *cdc2* mRNA was used as an internal loading control. The mRNA signal for *ura4* was produced by the *KΔ ::ura4* locus as the indigenous *ura4* allele was deleted in SPG74.

1988). This phenotype is expected for cells if they express both *P* and *M* information in haploid cells (Thon and Klar, 1992). Second, we did not observe mRNA specific to donor loci in nonswitching strains by Northern analysis (data not shown). Although donor loci remained silent, the *KΔ ::ura4* cells showed interesting covariegation of *ura4* expression and efficiency of mating-type interconversion. Cells from a single colony of SPG27, showing Ura$^+$ phenotype, were grown on rich medium and plated on medium lacking uracil (URA$^-$) or on counterselective medium containing 5′-fluoroorotic acid (FOA). The *ura4* gene product is required for growth on URA$^-$ minimal medium. In contrast, only cells with a repressed or mutated *ura4* gene can grow in the presence of FOA, because cells with a functional *ura4* gene convert FOA into a toxic metabolite (Boeke et al., 1987). Although all cells were genetically identical, we observed colonies of the SPG27 strain with predominantly two distinct phenotypes. A majority of the cells were capable of forming colonies on the URA$^-$ medium and, as expected, failed to grow on FOA plates. However, a small proportion (approximately 1%) were capable of growing on FOA plates, indicating that *KΔ ::ura4* expression in these cells was repressed (see below).

To test the relationship of the Ura phenotype with the mating-type switching, we determined the efficiency of mating-type interconversion in Ura$^+$ and Ura$^-$ cells by pedigree analysis of haploid cells as well as by iodine staining of individual colonies, an assay indicating the efficiency of switching at the colony level (Moreno et al., 1991). We found that Ura$^+$ SPG27 cells switched to the opposite mating type in approximately 30% (19 of 64) of cell divisions, in contrast to the rate of 80% (104 of 130) observed in wild-type cells, and hence stained lightly with iodine vapors (see below). In comparison, Ura$^-$ (i.e., FOA–resistant) colonies stained darkly with iodine and switched in approximately 65% (47 of 72) of cell divisions. The Ura$^+$ light-staining and Ura$^-$ dark-staining phenotypes henceforth will be referred to as light and dark forms, respectively.

To test directly whether the Ura$^+$ and Ura$^-$ phenotypes were the result of differences in *ura4* expression, we performed Northern analysis. The results (Figure 1) demonstrated, first, that *ura4* mRNA levels were negligible in cells showing the dark phenotype, but in light cells the levels were roughly comparable to those of the Ura$^+$ control cells in which the mRNA had been derived from the indigenous *ura4* gene. Second, both forms maintained their respective level of mRNA expression under nonselective (YEA) growth conditions. Third, light and dark cultures derived from cells of opposite phenotypes reflected levels of *ura4* mRNA indicative of the new form. We concluded that observed differences in the growth of light and dark cells on FOA and URA$^-$ media were due to differences in the level of *ura4* gene expression and that *ura4* expression and staining phenotypes were correlated.

Dark and Light States Are Metastable

It was possible that rearrangements in the *mat* region could occur at an increased rate in *KΔ ::ura4* cells, resulting in variegated phenotypes. However, Southern analysis showed that the difference between dark and light forms was not attributable to rearrangements in the *mat* region (data not shown). We next addressed

Table 1. S. pombe Strains Used in This Study

Strain Number	*mat* Region	Genotype
SP1000	h^{90}	*swi6-115, leu1-32, ura4, his2, ade6-210*
SPG16	h^{90}, *KΔ::ura4*	*swi6-115, leu1-32, ura4, his2, ade6-210*
SPG27	h^{90}, *KΔ::ura4*	*leu1-32, ura4, his2, ade6-210*
SPG51	h^{90}, *KΔ::ura4*	*leu1-32, ura4, ade6-216*
SPG60	h^{90}, *KΔ::ura4*	*clr1-5, leu1-32, ura4, his2, ade6-216*
SPG62	h^{90}, *KΔ::ura4*	*clr3-735, leu1-32, ura4, his2, ade6-210*
SPG64	h^{90}, *KΔ::ura4*	*clr4-681, leu1-32, ura4, his2, ade6-210*
SPG66	h^{90}, *KΔ::ura4*	*clr2-760, leu1-32, ura4, his2, ade6-216*
SPG74	h^{90}, *KΔ::ura4*	*leu1-32, ura4-D18, his2, ade6-210*

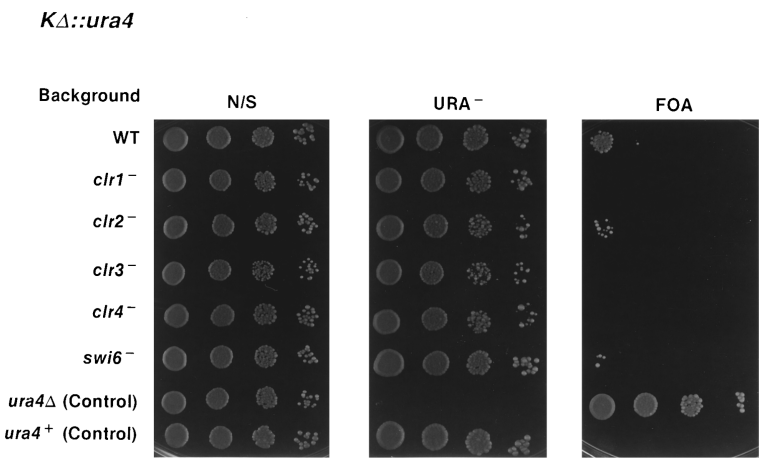

Figure 2. Mutations in *Trans*-acting Functions Affect *K∆ ::ura4* Expression

ura4 expression was measured as described in Thon and Klar (1992). Cultures grown to log phase in N/S (non/selective, EMM) medium were suspended in water. Serial dilutions (10-fold) of cell suspensions were spotted onto nonselective URA$^-$ (EMM minus ura), and FOA (EMM plus FOA) media, and plates were incubated at 33°C for 3–4 days. A strain containing the functional *ura4* gene at the wild-type chromosomal location and a strain in which *ura4* was deleted were used as controls. Strains used were: SPG27 (WT), SPG60 (*clr1*$^-$), SPG66 (*clr2*$^-$), SPG62 (*clr3*$^-$), SPG64 (*clr4*$^-$), and SPG16 (*swi6*$^-$). All strains containing *K∆ ::ura4* carried the mutation or deletion of the indigenous *ura4* gene. WT indicates wild-type genotype.

whether the dark and light phenotypes resulted from variegation controlled by an epigenetic mechanism rather than conventional mutational alterations. If mutational alterations were responsible for the two phenotypes, they would be expected to be extremely stable and interconvert at a very low rate. However, if variegation resulted from epigenetic changes, then phenotypes might be expected to interconvert at rates significantly higher than the rate of conventional genetic mutation. Therefore, we quantitated mitotic stability of both forms by using two different assays. In the first assay, the dark and light forms were allowed to grow nonselectively for approximately 25 generations on rich medium, diluted, and plated onto URA$^-$ and FOA medium, respectively. We found that both forms were mitotically metastable and that approximately 1%–2% of the cells in each case had converted to the opposite state. The newly converted forms were similarly mitotically metastable and changed to the opposite state at a frequency similar to that of their parents. Similar results were also obtained when stability of light and dark forms was tested using sporulation assay on nonselective medium (PMA$^+$). These results showed that changes in phenotypes occurred in both forms and at a frequency considerably higher than that expected for a conventional spontaneous mutation and that an epigenetic-based mechanism rather than genetic-based change was responsible for the light and dark phenotypes.

Mutations in *Trans*-Acting Regulators of *mat2* and *mat3* Silencing Suppress Variegation

Since products of the *clr1*, *clr2*, *clr3*, *clr4*, and *swi6* genes have been implicated in silencing of donor mating-type loci as well as that of the *ura4* gene placed adjacent to these loci, we tested whether mutations in these loci affect variegation of the *K∆ ::ura4* expression. We included strains containing *K∆ ::ura4* in the wild-type background or carrying a normal copy of *ura4* or mutated *ura4* gene as controls. Figure 2 shows the growth of these strains on indicated media. *K∆ ::ura4* in the wild-type background supported the growth of approximately 99% of the cells on URA$^-$ plates and formed only a few colonies on FOA plates. Mutations in all *trans*-acting genes impaired the ability of *K∆ ::ura4* cells to form colonies on FOA plates, suggesting further derepression of the *ura4* reporter gene. These results suggest that the products of these genes influence variegation of *K∆ ::ura4* expression. The observed growth of colonies on FOA plates in the *clr2* and *swi6* mutant backgrounds was mainly due to the rearrangements in the mating-type region that generated *h*$^{-S}$ cells (Beach and Klar, 1984). Such rearrangements are known to occur more frequently in *swi6*$^-$ cells (Lorentz et al., 1992).

Dark and Light States Are Chromosomally Inherited During Mitosis and Meiosis

In principle, epigenetic control might operate by chance variations of some critical protein concentration or by mediating chromosomally inherited imprinted events but without alterations of DNA itself. We differentiated between these models by following genetic crosses. Two closely related *K∆::ura4* strains, SPG51(D) (D, dark) and SPG27(L) (L, light), showing dark and light phenotypes, respectively, were mated to construct a diploid. The diploid was allowed to grow for at least 30 generations, sporulated, and subjected to tetrad analysis. If the dark and light states were meiotically stable as well as chromosomally inherited both during mitosis and meiosis, then two Ura$^-$ dark-staining and two Ura$^+$ light-staining meiotic segregants should be found in each tetrad. Furthermore, if these states were inherited through the *K*-region, they should cosegregate with the closely linked (1 cM) *his2* marker. Since the mating-type region of SPG27(L) was marked with the mutation in *his2* gene, Ura$^+$ light-staining segregants should not grow on media lacking histidine (HIS$^-$). Remarkably, such a segregation pattern was found in all 25 tetrads analyzed, 10 of which are shown in Figure 3. We next reversed the phenotypes of SPG51(D) and SPG27(L) by selecting for the growth of their cells on URA$^-$ and FOA media, respectively. The resultant strains, SPG27(D) and SPG51(L), showing dark and light phenotypes, respectively, were crossed. Among the 18 tetrads analyzed, 2:2 segregation of dark and light states and their linkage to *his2* locus (data not shown) confirmed the results obtained in the first cross. Significantly, both crosses showed that dark and light variegated states were linked to the mating-type region and were chromosomally inherited in meiosis as a conventional genetic marker.

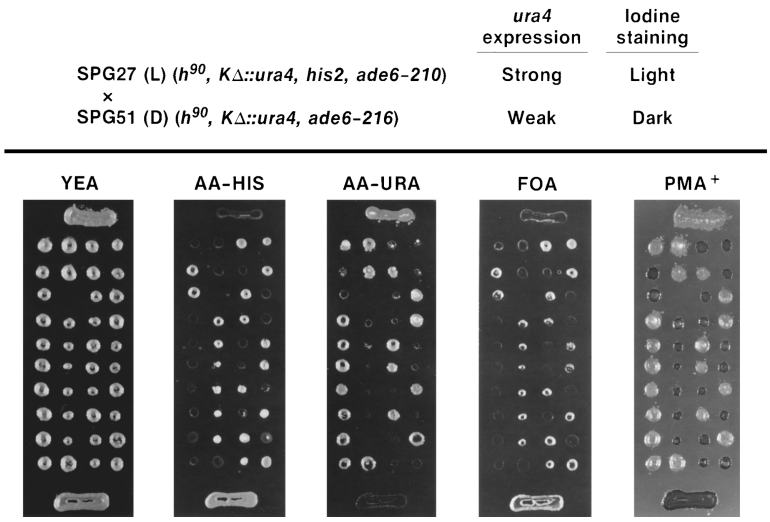

Figure 3. Light and Dark Variegated States Are Meiotically Stable and Linked to the *mat*-Region

A diploid was constructed by crossing the indicated strains, allowed to grow for at least 30 generations, sporulated, and subjected to tetrad analysis. The four spores from each ascus were placed on rich medium in a horizontal row by micromanipulation. After growth for 3 days, they were replicated onto plates containing the indicated medium. All plates were photographed directly after overnight growth at 33°C except for the PMA^+ plate, which was incubated for 3 days at 25°C and stained with iodine vapors before photography. Each ascus produced two Ura^+, His^- light-staining, two Ura^-, His^+ dark-staining segregants. Patches at the top and bottom indicate the phenotype of the parental SPG27(L) and SPG51(D) strains, respectively, used in the cross.

These results clearly showed that during mitotic growth of the diploid, each chromosome maintained its respective variegated state for at least 30 generations. Therefore, we conclude that dark and light states in mitotically growing cells are chromosomally inherited. Further supporting this conclusion, we similarly found that respective variegated states were linked to *KΔ ::ura4* when crossed with a wild-type *K*-region–containing strain (data not shown). Also, meiotic segregants of either form exhibited similar metastability.

Discussion

In this paper we demonstrate that, first, an epigenetic mechanism regulates the efficiency of mating-type interconversion and silencing of a marker gene inserted at the *K*-region; second, generation, or propagation, or both, of these epigenetic states is shown to be under the control of *clr1*, *clr2*, *clr3*, *clr4*, and *swi6* gene functions; and third, epigenetic states are remarkably stable and are inherited during mitosis and meiosis as Mendelian genetic markers.

Light and Dark Epigenetic States Presumably Reflect Change in Chromatin Organization

We have shown that donor loci remain silent in *KΔ ::ura4* cells. This finding suggests that remaining sequences around the silent mating-type loci in *KΔ ::ura4* cells are sufficient to keep them in the repressed state. However, our recent data suggest that the deleted part of the *K*-region, which shares homology with S. pombe centromeric sequences, plays a role in silencing of donor loci, but the effect of the deletion is not visible owing to redundancy in silencing pathways (our unpublished data). Most importantly, we demonstrated that an epigenetic effect regulates both the expression of *KΔ ::ura4* and the efficiency of mating-type interconversion. This epigenetic effect results in production mainly of two variegated forms, called light and dark epistates. The correlation between silencing of *KΔ ::ura4* and the ability to switch efficiently in dark cells suggests that donor choice and silencing are controlled by the same mechanism. Previous work has suggested that chromatin structure plays a role in donor choice and silencing (Klar, 1992; Thon and Klar, 1993). According to this model, the repression of *ura4* and the increased frequency of heterologous mating-type switching in dark cells might be the result of specific chromatin organization of the mating-type region, with light cells being defective in assembly of the proposed chromatin structure. The following observations support the chromatin assembly model for explaining the light and dark phenotypes. First, light and dark epigenetic states share many similarities with position-effect variegation in Drosophila, where heterochromatin-mediated transcriptional repression results in inactivation of a particular gene in some cells but not in other cells (see Eissenberg et al., 1995). Second, mutations in *clr1*, *clr2*, *clr3*, *clr4*, and *swi6* genes, whose wild-type forms are proposed to assemble silent mating-type loci, centromeres, and telomeres in heterochromatin-like structures (Klar and Bonaduce, 1991; Thon and Klar, 1992; Ekwall and Ruusala, 1994; Thon et al., 1994; Allshire et al., 1995), suppress variegation of *KΔ ::ura4* expression. Third, Swi6 (Lorentz et al., 1994) and Clr4 (A. Ivanova, M. Bonaduce, and A. J. S. K., unpublished data) proteins contain a 37 amino acid "chromodomain" motif found in chromatin-associated proteins such as HP1 and Polycomb proteins of Drosophila; M31, M32, and M33 proteins of mouse; and the human HSM1 protein (reviewed by Eissenberg et al., 1995).

The model unifying the information discussed above states that *clr1–clr4* and *swi6* gene products act through the entire *mat2–mat3* interval, including the *K*-region, to assemble a heterochromatin-like structure that prohibits transcription and meiotic recombination in this region. Also, this structure is required to make the donor loci readily accessible to *mat1* for switching in a cell-type–specific manner. This process apparently occurs readily in all cells when the entire *cis*-acting information is present, but in *KΔ ::ura4* cells, we propose that the minimized DNA structures needed for action of the *trans*-acting factors compromise the formation of the stable heterochromatin-like structure, resulting in a variegated phenotype.

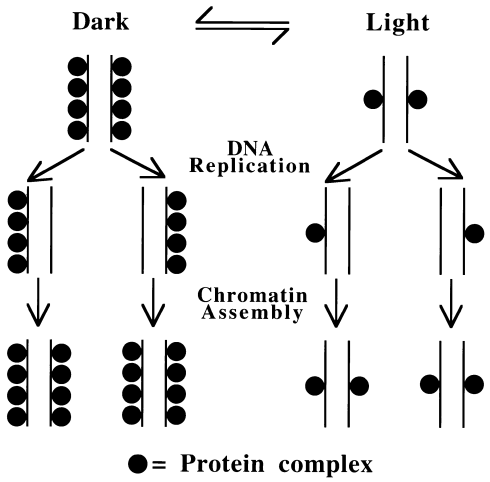

Figure 4. A Hypothetical Chromatin Replication Model Explaining Chromosomal Inheritance of the Light and Dark Epigenetic States

Formation of sufficient nucleoprotein complexes at the *mat2–mat3* interval results in dark form, while light form is deficient in such complexes. Cooperative binding of these proteins, which might not be completely displaced during DNA replication, results in inheritance of respective states by daughter chromosomes.

A Hypothetical Chromatin Replication Model for Inheritance of Epistates

Meiotic stability of epigenetic states implied that, unlike what was suggested for Maize (McClintock, 1987), these epigenetic states were not "erased" or "reset" during meiosis. Most importantly, we found that light and dark states were property of the locus rather than the result of change in some other component of the cell, since in diploids each chromosome maintained its respective state for as many as 30 generations. These data imply that the *mat2–mat3* interval is imprinted or marked in such a way that, once established, the epigenetic alterations defining each state must be capable of self-replicating during DNA synthesis.

At present, we can only speculate on the nature of the imprinting event. This event might be due to DNA methylation, chromosome topology, covalent modification of DNA strands, nuclear compartmentalization, or specific timing of replication in the cell cycle. Thus far, no DNA modification has been observed in S. pombe (Antequera et al., 1984, J. Kohli, personal communication). At present, we favor a chromatin-assembly model in which a nucleoprotein complex that is semiconservatively segregated to each strand during replication can promote its reassembly in a self-templating manner (Figure 4). In this model, we propose that *cis*-acting regulatory sequences within the *mat2–mat3* interval serve as anchors for proteins such as Clr1–Clr4 and Swi6, which either bind directly to DNA or form complexes through protein–protein interactions. Once a structure representing a particular epigenetic state is formed, it might split during replication, segregating copies of the protein complexes to both daughter chromatids as hypothesized earlier (Brown, 1984; Wolffe, 1994). These nucleoprotein complexes could then directly sequester additional protein factors by a form of cooperative binding, resulting in replication of chromatin on both chromatids. Localization of the *mat* region in a specific compartment of the nucleus may facilitate this process, for example, because of an environment enriched for proteins required for silencing. In this model, DNA replication would provide a window of opportunity for the formation of new nucleoprotein complexes or dissociation of existing complexes, resulting in a change of one epigenetic state to another (Klar, 1994). In our model, we expect that overexpression of some genes such as *clr1*, *clr2*, *clr3*, *clr4*, or *swi6* would lead to an increased proportion of cells exhibiting the dark phenotype. Our recent analysis showed that multiple copies of *clr4* as well as *swi6* genes indeed transformed the *KΔ ::ura4* cells exhibiting light phenotype into the dark form (our unpublished data).

Implications of Chromosomal Inheritance of Epigenetic States in Development

Chromosomal inheritance of epigenetic states in mitosis and meiosis has wide-ranging implications in development. Analogous to the stable meiotic inheritance of light and dark epigenetic states, the *trans*-inactivated allele in Huntington's disease has been proposed to persist occasionally through meiosis (Sabl and Laird, 1992). Other closely related examples sharing some parallels with the phenomenon described above include the state of repression of *HMLα* in *sir1* mutants of S. cerevisiae (Pillus and Rine, 1989), inactivation of X chromosomes in mammals (reviewed by Grant and Chapman, 1988), and position-effect variegation in Drosophila (see Eissenberg et al., 1995). Although repression of *HMLα* in the *sir1* background was inherited clonally, in contrast to our results, repression was found to be the property of a cell rather than being inherited through the *HML* locus itself (Pillus and Rine, 1989). Based on the clonal transmission of the heterochromatin-mediated repression and the repressed states of homeotic regulatory genes during development in Drosophila, Paro (1990) proposed that the architecture of chromatin surrounding the gene, rather than diffusible factors, serves as a cellular memory transmitting the determined state of a cell to its mitotic descendants. However, chromosomal inheritance of cellular memory in most of these cases remains to be directly demonstrated. In contrast to meiotic stability of epigenetic states in our system, such states must be erased during meiosis in the aforementioned systems. As for meiotic stability, more analogous situations to our system comprise the phenomena of paramutation at the b locus (Patterson et al., 1993) and methylation-correlated epigenetic control observed in Ascobolus, Arabidopsis, Neurospora, and Maize (Brutnell and Dellaporta, 1994; Bender and Fink, 1995; Colot and Rossignol, 1995; Singer and Selker, 1995).

Although our observation of meiotic inheritance of epigenetic states is novel, we imagine that such a mechanism may be operative to prohibit wasteful transcription of noncoding sequences adjacent to fortuitous promoter elements and sequences in telomere and centromeres, as well as for repression of deleterious transposable elements prevalent in the eukaryotic genome. Furthermore, the meiotic stability of epigenetic states has allowed us to discover chromosomal inheritance of such a control of gene expression in mitotically dividing

cells. We imagine that chromosomal inheritance of states of gene expression in mitotically dividing cells is likely to be prevalent and essential for cellular differentiation in development. The study of already identified genes that affect variegation of *KΔ ::ura4* expression would provide us with a unique tool for investigating the phenomenon in molecular terms.

Experimental Procedures

Plasmid Constructions

All cloning steps were performed according to standard techniques (Sambrook et al., 1989). pSG2, the 2.1 kb BamHI–HindIII fragment from pSP10 (Kelly et al., 1988), was subcloned into BamHI–HindIII restriction sites of pWH5. Subsequently, the 1.5 kb HaeIII–HindIII fragment from pSM10 (Kelly et al., 1988) was inserted into SmaI–HindIII sites (pSG1). The 1.8 kb HindIII restriction fragment containing the S. pombe *ura4* gene was inserted into the HindIII site of pSG1.

Strains and Culture Conditions

All strains used in this study were constructed in this laboratory, and genotypes are listed in Table 1. Standard conditions for growth and genetic analysis were as described by Moreno et al. (1991). As a result of a recombination block in the *mat2–mat3* interval as well as the silencing mechanism operative in the *K*-region (Egel, 1984), targeted integration of sequences in this region by DNA–mediated transformation is difficult to achieve. To overcome this problem, replacement of part of the *K*-region with *ura4* was performed in the *swi6* mutant background, which allows recombination in this region and also allows expression of *ura4* inserted there (Thon et al., 1994). Transformants with integration at the desired site were identified by Southern analysis of DNA isolated from several stable Ura^+ transformants. SPG16 was constructed by transforming SP1000 with 5.4 kb BamHI–NdeI fragment from pSG2. Standard genetic crosses were used to construct all other strains.

DNA and RNA Analysis

DNA from overnight-grown S. pombe cultures was prepared as described in Moreno et al. (1991), and RNA was prepared essentially as described in Schmitt et al. (1990). Southern and Northern hybridizations were carried out as described in Sambrook et al. (1989).

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